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Dental Caries in Rats Associated with *Candida albicans*

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Key Words
Candida albicans · Confocal · Dental caries · Microscopy · Rats · *Streptococcus mutans*

Abstract
In addition to occasional opportunistic colonization of the oral mucosa, *Candida albicans* is frequently found in carious dentin. The yeast's potential to induce dental caries as a consequence of its pronounced ability to produce and tolerate acids was investigated. Eighty caries-active Osborne-Mendel rats were raised on an ampicillin-supplemented diet and exposed to *C. albicans* and/or *Streptococcus mutans,* except for controls. Throughout the 28-day test period, the animals were offered the modified cariogenic diet 2000a, containing 40% various sugars. Subsequently, maxillary molars were scored for plaque extent. After dissection, the mandibular molars were evaluated for smooth surface and fissure caries. Test animals exposed to *C. albicans* displayed considerably more advanced fissure lesions ($p < 0.001$) than non-exposed controls. While *S. mutans* yielded similar results, a combined association of *C. albicans* and *S. mutans* had no effect on occlusal caries incidence. Substituting dietary sucrose by glucose did not modify caries induction by *C. albicans*. However, animals fed a diet containing 20% of both sugars showed no differences to non-infected controls. Smooth surface caries was not generated by the yeast. This study provides experimental evidence that *C. albicans* is capable of causing occlusal caries in rats at a high rate.

Due to its potential to cause superficial and systemic infections in immuno-compromised patients, the yeast *Candida albicans* is classified as a human pathogen. One of the preferred locations of *C. albicans* in healthy individuals is the oral cavity, where it colonizes mucosal sites as a commensal, but may engender candidiasis in cases with predisposing local host factors [Odds, 1988]. However, the predominant oral habitats of *C. albicans* in children are carious lesions, from which this fungus has been isolated with high frequencies (up to 97%) [Schulz-Weidner et al., 2005]. A strong association has been found between the prevalence of *C. albicans* and dental caries – particularly in children, adolescents and young adults [Gabris et al., 1999; Moalic et al., 2001; Beighton et al., 2004]. These epidemiological data suggest that *C. albicans* could play an active role in the pathogenesis of caries.

There are several characteristics of this microorganism that are related to cariogenicity. The fungal H^+-ATPase, which actively pumps protons out of the cell [Bowman and Bowman, 1986], causes an extraordinarily

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high acid tolerance and enables rapid extracellular acidification. In addition, *C. albicans* excretes organic acids, mainly pyruvic ($pK_a = 2.39$) and acetic ($pK_a = 4.75$) acid [Klinke et al., 2009], the former being even more potent than lactic acid ($pK_a = 3.86$) in decreasing the pH of an already intensely acidified environment. Investigations into the velocity of acid formation from glucose by *C. albicans* and oral lactobacilli at pH-stat conditions revealed a similar acidogenicity of both microorganisms [Klinke et al., 2009].

Furthermore, *C. albicans* adheres to saliva-coated hydroxyapatite [Cannon et al., 1995] and binds to native or denatured collagen [Makihira et al., 2002]. Fungal secreted aspartyl protease was shown to be capable of degrading dentinal collagen under acidic conditions [Hagihara et al., 1988; Klinke et al., 2007].

Despite the fact that the features described above indicate *C. albicans* is a potent caries pathogen, its significance has been often denied since colony-forming units of fungi usually represent only a small percentage of the total microbiota. On the other hand, the biomass of *C. albicans* is much larger than those of bacteria like streptococci [Klinke et al., 2009].

The aim of the animal study reported here was to investigate whether *C. albicans* could induce dental caries in vivo. The diet's influence (glucose and/or sucrose) and the effects of co-association with *S. mutans* were also studied.

Materials and Methods

Strains and Inoculum Preparation

Streptococcus mutans UA159 (ATCC 700610) was obtained from the American Type Culture Collection and *C. albicans* OMZ 1067 was isolated from human carious dentin. Strains were maintained by anaerobic *(S. mutans)* or aerobic *(C. albicans)* incubation at 37°C on plates of Columbia Blood Agar Base (Oxoid, Ltd., Basingstoke, UK) supplemented with 5% (v/v) hemolyzed human blood (CBA). Loopfuls of Columbia Blood Agar Base-grown cells were inoculated into filter-sterilized fluid universal medium [Gmur and Guggenheim, 1983] supplemented with 67 mmol/l Sorensen's buffer, pH 7.2 ('modified fluid universal medium', mFUM). Pre-cultures (10 ml) in mFUM were incubated anaerobically *(S. mutans)* or aerobically *(C. albicans)* at 37°C for 15 h; cultures of each species were prepared by inoculation of mFUM (50 ml) with aliquots (1 ml) of pre-culture and incubation at 37°C for ca. 7 h or until $OD_{550} \geq 1.0$ ($\sim 10^7$ cells/ml). In order to ensure implantation of the microorganisms, a cell culture concentration of approximately 10^8 cells is required [van Houte et al., 1976]. Therefore, following microscopic verification of purity, inocula were prepared by 10-fold concentration of the microbial cultures using fresh mFUM.

Animals and General Procedures

The animal study, conforming to the Swiss animal protection laws and approved by the Veterinäramt des Kantons Zürich, was conducted on 10 litters of caries-active Osborne-Mendel rats, each litter consisting of 8 pups. Thirteen days after birth, the pups and their dams were transferred to stainless steel screen-bottom cages without bedding and fed a finely ground stock diet (diet No. 3433, Provimi Kliba AG, Switzerland) in order to prevent impaction of food and bedding particles in fissures. Up to day 23 after birth, 100 ml of water supplemented with 1 $mg \cdot ml^{-1}$ ampicillin was administered to the animals in order to suppress the oral microbial flora and to facilitate the association of the test species. Additional tap water was given ad libitum. On day 23, the dams were removed. Oral swabs using sterile cotton buds were taken from the dams and evaluated microbiologically in order to exclude any previously existing infections by the test species. The 80 littermates were distributed at random among 8 treatments (table 1), 2 animals per cage, in a complete-block design. From day 23 until the end of the 28-day study period, the rats were offered the modified cariogenic diet 2000a [Guggenheim et al., 1966] containing 40% varying sugars (table 1), 28% skim milk, 24% wheat flour, 5% brewer's yeast, 2% Gevral protein (Whitehall-Robins, Zug, Switzerland) and 1% NaCl. Drinking water was available ad libitum.

Microbial Association and Analyses

Twice daily on days 24 and 25, the rats were orally administered 200 μl of the dense microbial suspension of *C. albicans* OMZ 1067, isolated from human carious dentin, and/or *S. mutans* UA159 (table 1), using a tuberculin syringe without a needle. Negative controls received water. The establishment of the test strains was verified 6 days later. During these 2 days, drinking water additionally contained 2% glucose and 2% sucrose.

At the end of the study on day 53, the microbiological status of the animals was evaluated. Oral swabs were taken, suspended in 5 ml of reduced transport fluid [Loesche et al., 1972] and 50-μl aliquots were dispensed with a spiral diluter (Spiral Systems Inc. Cincinnati, Ohio, USA) onto agar plates. Blood agar plates (Difco, No. 279240, BD Diagnostic Systems, Franklin Lakes, N.J., USA) containing 5% hemolyzed human blood were used to evaluate the total number of culturable microorganisms (colony-forming units). *S. mutans* colonies were counted on TSY20B agar [Schaeken et al., 1986] supplemented with 200 $IU \cdot l^{-1}$ bacitracin and *C. albicans* on BIGGY Agar (BBL, No. 211027, BD Diagnostic Systems). Mitis-Salivarius agar (Difco, No. 229810, BD Diagnostic Systems) with 10 $mg \cdot l^{-1}$ potassium tellurite [MacFaddin, 1985] was used to determine the total number of streptococci. Kanamycin-esculin-acide agar (Merck No. 1.05222.0500) was used to evaluate enterococcal infection. Bacteria were cultured in a CO_2 atmosphere for 72 h at 37°C, while yeasts were incubated aerobically at 37°C for 24 h.

Plaque and Caries Evaluation

On day 53, the animals were anaesthetized with CO_2 and decapitated. The upper and lower jaws were dissected and immersed in 10% phosphate-buffered formalin for a minimum of 72 h. Erythrosine-stained maxillary molars were scored for the extent of smooth surface plaque using the method described by Regolati and Hotz [1972]. The mandibular molars were evaluated for buccal smooth surface caries [Keyes, 1958] and fissure caries

Table 1. Colony-forming units of microorganisms recovered from oral swabs taken from rats fed with a sucrose- and/or glucose-containing cariogenic diet and associated with *C. albicans* or *S. mutans*, or both, 28 days after association

Treatment group	Sugars in diet 2000a	Associated strains	Microorganisms recovered from oral swabs lg (colony-forming units ± SD)			
			total	Streptococci	*S. mutans*	*C. albicans*
1	40% sucrose	none	7.84 ± 0.47	4.99 ± 0.37	0 ± 0	0 ± 0
2	40% sucrose	*C. albicans*	7.74 ± 0.19	4.39 ± 1.56	0 ± 0	1.70 ± 1.52
3	40% sucrose	*S. mutans*	8.38 ± 0.13	7.80 ± 0.44	7.68 ± 0.24	0 ± 0
4	40% sucrose	*C. albicans* + *S. mutans*	8.17 ± 0.22	7.34 ± 0.53	7.20 ± 0.52	3.66 ± 0.72
5	40% glucose	none	7.64 ± 0.29	4.83 ± 0.45	0 ± 0	0 ± 0
6	40% glucose	*C. albicans*	7.79 ± 0.36	4.94 ± 0.31	0 ± 0	1.63 ± 1.44
7	20% sucrose + 20% glucose	none	7.76 ± 0.25	4.32 ± 1.55	0 ± 0	0 ± 0
8	20% sucrose + 20% glucose	*C. albicans*	7.84 ± 0.26	4.47 ± 1.59	0 ± 0	2.56 ± 1.07

Each treatment group consisted of 10 animals. Associations (*C. albicans* OMZ 1067 and *S. mutans* OMZ 918) were administered 4 times.

[König et al., 1958]. The rats were weighed at the beginning and end of the study, and their weight gains calculated. All other minor details of the experimental procedures have been described and reviewed by König [1966].

Statistical Analyses

Each treatment consisted of 10 animals. Data of treatments 1–4 were subjected to a two-way ANOVA with Bonferroni correction. Treatments 5–8 were analyzed by unpaired t tests. Statistical procedures were performed with StatView 5.01 (SAS Institute, Cary, N.C., USA).

Fluorescent in situ Hybridization

For the purpose of fluorescent in situ hybridization (FISH) and confocal laser scanning microscopy (CLSM), the upper jaws were demineralized in buffered EDTA, embedded in epoxy resin (Technovit 9100, Heraeus Kulzer, Wehrheim, Germany) and cut into sections of 5- to 10-μm thickness. The epoxy resin was removed and the sections were fixed on silane-coated microscope slides.

A custom-synthesized *C.-albicans*-specific 18S rRNA probe CAAL1249 [Kempf et al., 2000], labeled at the 5′-end with Cy5, was purchased from Microsynth (Balgach, Switzerland). The probe was used at a final concentration of 5 ng · ml^{-1} in the presence of 30% formamide in the hybridization buffer. FISH was performed in 50-ml plastic centrifuge tubes at 46°C as described [Thurnheer et al., 2001] except for the following modifications [Gmur and Luthi-Schaller, 2007]: (1) the slides were not dehydrated in ethanol prior to hybridization; (2) sections were covered for 60 min at 37°C with protectRNA RNase inhibitor (Sigma-Aldrich, St. Louis, Mo., USA, diluted 1:500 in 0.9% NaCl) prior to hybridization.

CLSM and SEM

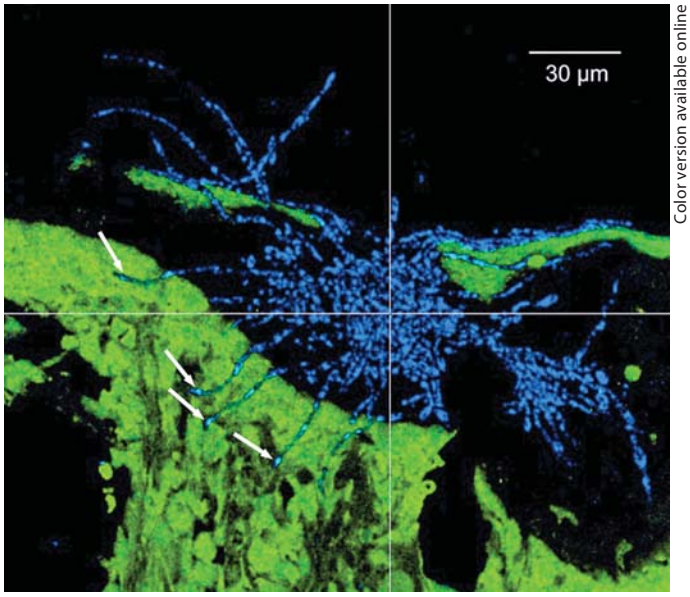
FISH-stained sections were examined using a DM IRB/E inverted microscope (Leica Mikroskopie GmbH, Wetzlar, Germany) fitted with a UV laser (Coherent Inc., Santa Clara, Calif., USA), a He-Ne laser (Uniphase Vertriebs GmbH, Eching/Munich, Germany), an Ar laser (Coherent Inc.) and a TCS SP5 computer-operated confocal laser scanning system (Leica Lasertechnik GmbH, Heidelberg, Germany). Filters were set to 500–540 nm to detect dentin autofluorescence and to 660–710 nm for Cy5. Confocal images were obtained using a ×63 (numeric aperture 1.30) glycerol immersion objective. Z-series were generated by vertical optical sectioning with the slice thickness set at 1.018 μm. Image acquisition was performed in ×8 line average mode and the data were processed on a Silicon Graphics 320™ visual workstation (Mountain View, Calif., USA) fitted with Windows NT v4.0. Scans were recombined using Imaris™ 3.1 software (Bitplane AG, Zürich, Switzerland).

SEM images were obtained from upper molars after longitudinal dissection. The samples were air-dried, gold-sputtered and examined with a Philips (Eindhoven, The Netherlands) XL30 SEM at an accelerating voltage of 20.0 kV.

Results

At the end of the experimental period, all animals were in good health and showed no significant differences in weight gains. The microorganisms *C. albicans* and *S. mutans* had colonized the oral cavities of the respective animals, as proven by the oral swabs taken at the end of the study (table 1). No cross-contamination between animals of different treatment groups occurred.

Co-association of *S. mutans* and *C. albicans* in treatment group 4 resulted in decreased counts of mutans streptococci ($p < 0.01$), but raised counts of the yeast ($p < 0.001$) compared to the corresponding mono-exposure



Color version available online

Fig. 1. CLSM image of *C. albicans* penetrating dentinal tubules of a mandibular molar (arrows) in treatment 4. FISH staining was accomplished using probe CAAL1249 labeled with Cy5. Dentin is also visible due to autofluorescence.

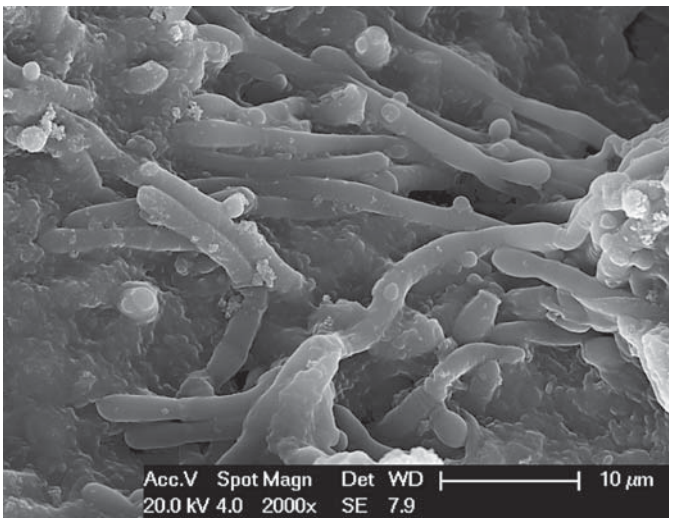


Fig. 2. SEM image showing the biofilm in an occlusal fissure (treatment 4) colonized by *C. albicans* in budding and hyphal forms.

Table 2. Mean and standard deviation of plaque extent and caries incidence in rats fed with sucrose- and/or glucose-containing cariogenic diet and associated with *C. albicans* or *S. mutans* or both

Treatment group	Sugars in diet 2000a	Associated strains	Plaque extent[a]	Initial dentinal fissure lesions[b]	Advanced dent. fissure lesions[b]	Smooth surface caries lesions[c]
1	40% sucrose	none	1.2 ± 0.42	10.1 ± 0.99	6.5 ± 2.59	0.1 ± 0.32
2	40% sucrose	*C. albicans*	1.5 ± 0.53, n.s.	11.3 ± 0.94, n.s.	11.0 ± 1.05***	1.4 ± 2.79, n.s.
3	40% sucrose	*S. mutans*	2.0 ± 0.47***	11.5 ± 1.27, n.s.	10.8 ± 1.62***	7.5 ± 3.17***
4	40% sucrose	*C. albicans* + *S. mutans*	1.7 ± 0.48, n.s.	11.4 ± 1.35, n.s.	4.2 ± 2.44, n.s.	6.0 ± 4.80**
5	40% glucose	none	1.0 ± 0.00	9.3 ± 2.83	4.6 ± 2.12	0.2 ± 0.63
6	40% glucose	*C. albicans*	1.1 ± 0.32, n.s.	11.1 ± 0.88, n.s.	10.0 ± 0.82***	0.2 ± 0.42, n.s.
7	20% sucrose + 20% glucose	none	1.7 ± 0.82	7.1 ± 2.72	3.0 ± 1.83	0.4 ± 0.97
8	20% sucrose + 20% glucose	*C. albicans*	1.4 ± 0.52, n.s.	8.9 ± 2.47, n.s.	2.5 ± 1.84, n.s.	0.3 ± 0.95, n.s.

Each treatment group consisted of 10 animals. Asterisks indicate significant differences between test treatments and the respective non-associated controls fed on the same diet. [a]4 units at risk; [b]12 fissures at risk; [c]20 units at risk. n.s. = Nonsignificant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

treatments 3 and 2, respectively (table 1). The extent of smooth surface plaque was increased by the association of *S. mutans,* but not by *C. albicans* (table 2).

Rats exposed to *C. albicans* in the 40% sucrose group (treatment 2) showed considerably more advanced fissure lesions ($p < 0.001$) than the controls (table 2). The association of *S. mutans* (treatment 3) gave comparable results with no statistical difference to the yeast. Surprisingly, the number of advanced lesions was not different from the controls if *C. albicans* and *S. mutans* were associated together in treatment 4. If sucrose in the diet was substituted by glucose (treatment 6), the incidence of advanced fissure lesions was increased by *Candida* infection in a similar manner; but in case of the combined 20% su-

crose/20% glucose diet (treatment 8) no difference could be observed compared to the non-infected controls. Smooth surface caries was found to be strongly enhanced by *S. mutans* ($p < 0.001$), while *C. albicans* was not able to induce this type of lesion.

Using CLSM, *C. albicans* was detected in both yeast and hyphal phenotypes colonizing occlusal fissures and penetrating the dentin via dentinal tubules (fig. 1). Scanning electron microscopy revealed the fungus in budding and hyphal growth forms, embedded in the bacterial biofilm (fig. 2).

Discussion

This study demonstrates for the first time that *C. albicans* is able to boost caries incidence if added to a mixed low-cariogenic microbiota in rats. A similar increase in advanced occlusal lesions was observed if the animals were fed diets containing either 40% sucrose or 40% glucose. Surprisingly, such an effect was missing when the diet combined both sugars at 20% each. From this carbohydrate mixture, the yeast was apparently not able to produce amounts of acid sufficient to cause caries, although *Candida* counts were comparatively high in the respective treatment. The reason for this phenomenon is likely to be based on the mechanisms of sucrose utilization in *C. albicans*. In contrast to *S. cerevisiae*, *C. albicans* does not exhibit invertase activity. Hydrolysis of sucrose is suggested to be performed by an α-glucosidase (maltase) following the transport of the disaccharide into the intracellular compartment via a sucrose-inducible permease [Williamson et al., 1993]. Geber et al. [1992] isolated the *C. albicans* α-glucosidase structural gene CAMAL2. Transcription of CAMAL2 was induced by both maltose and sucrose but repressed by glucose, as demonstrated by the lack of maltase mRNA in cells grown in a sucrose medium supplemented with glucose. Kelly and Kwon-Chung [1992] cloned the *C. albicans* gene CASUC1 which affects sucrose utilization and α-glucosidase activity. CASUC1 enabled the *S. cerevisiae* suc2 mutant devoid of invertase genes to utilize both sucrose and maltose. Addition of glucose to the growth medium completely inhibited α-glucosidase activity of this mutant. Along with the present study, the repressive effect of glucose on sucrose metabolism was investigated for the test strain *C. albicans* OMZ 1067 in vitro by measuring extracellular acid formation under pH-stat conditions using an automated titration system [Klinke et al., 2009]. Yeast cells grown in a medium containing 2% of both sucrose and glucose produced acid at a rate 10 times lower from sucrose than from glucose. If the washed cell suspension was titrated for a prolonged period, acid formation began to increase 10 min after the addition of sucrose and reached its maximum after 20 min, indicating a sucrose-induced expression of α-glucosidase in the absence of glucose. Based on these findings, it is reasonable that in treatment 8 almost exclusively glucose and hardly any sucrose was utilized by *C. albicans*. A 20% glucose diet, however, has to be regarded as rather low-cariogenic in the rat model [Guggenheim et al., 1997]. Still more than a prokaryotic microbiota, the yeast *C. albicans* is dependent on high concentrations of dietary sugars to produce acid. In vitro, starved cells of *C. albicans* required a 50-fold higher concentration of extracellular glucose than lactobacilli to enable half-maximum acid formation [Klinke et al., 2009]. Plaque bacteria like mutans streptococci and lactobacilli import mono- and disaccharides into the cell very quickly via the phosphoenolpyruvate-phosphotransferase system [Barrangou et al., 2006; Ajdic and Pham, 2007]. In contrast, glucose uptake in yeasts is achieved by an electrochemical gradient generated by the plasma membrane H^+-ATPase which is activated when glucose is present in the environment [Portillo, 2000]. *C. albicans*' contribution to caries progression may therefore depend on both the amount of fermentable carbohydrates and the proportion of sucrose and glucose in the diet.

Yeast counts in the test groups were low compared to streptococci or total bacterial microbiota. From oral swabs of some animals exposed to *C. albicans,* even no fungi could be isolated at all. This finding illustrates the weak ability of the yeast to colonize mucous membranes in healthy individuals and likewise smooth dental surfaces, which corresponds to the observed failure to produce carious lesions on the latter site. However, the low proportion of *C. albicans* does not reflect the composition of fissure plaque, where the yeast finds better conditions to colonize and to compete for this niche. In a clinical study of Sziegoleit et al. [2002], a considerably larger quantity of *C. albicans* was cultivated from carious material than from plaque or saliva samples of the same children. Examination of teeth acquired from treatment 4 using a scanning electron microscopic revealed extensive infiltration of fissure plaque by clusters of the fungus in both budding and hyphal forms (fig. 2). Additionally, comparing the numbers of colony-forming units between bacteria and yeasts with respect to their pathogenic impact is misleading as the cell sizes are distinctly different. The biomass of *C. albicans*, for example, is about 40 times larger than that of *S. mutans* [Klinke et al., 2009].

If co-associated with mutans streptococci, yeasts were increased significantly in number. This is most likely due to a lower environmental pH, where less acidotolerant bacteria are inhibited in growth and more aciduric species prevail. Less plausible would be a co-adhesion between these microorganisms observed in vitro. *C. albicans* proved able to bind to oral viridans streptococci and to *Actinomyces*, mediated by bacterial cell-wall polysaccharides, cell-surface proteins and salivary proline-rich proteins [O'Sullivan et al., 2000; ten Cate et al., 2009]. Regarding co-aggregation with mutans streptococci, published data are ambiguous. However, studies using saliva coated hydroxyapatite beads showed that pre-incubation with *S. mutans* did not reduce adhesion of *C. albicans* in contrast to *S. gordonii* or *S. oralis* [O'Sullivan et al., 2000], but increased the growth of a *Candida* biofilm in the presence of sucrose [Pereira-Cenci et al., 2008]. In gnotobiotic mice, a mixed salivary microbiota as well as pure cultures of *S. salivarius* or *S. mitis* restrained the colonization of *C. albicans* in the oral cavity, while *S. mutans* did not [Liljemark and Gibbons, 1973].

In conclusion, the present animal study provides experimental evidence that *C. albicans* is capable of causing advanced occlusal caries lesions at a high rate. This cariogenic potential, however, does not necessarily imply that dental plaque becomes cariogenic by *Candida* colonization. In view of the observed antagonistic effects, even caries-reducing interactions between yeasts and other caries-associated microorganisms are possible. Additional studies with biofilms are necessary to investigate the negative impact that co-association of *S. mutans* and *C. albicans* exerts on plaque cariogenicity. Nevertheless, the reputation of *C. albicans* as being an insignificant commensal of plaque microbiota is no longer tenable. Low numbers of yeasts in the oral cavity or on smooth dental surfaces provide no evidence for a negligible role of *C. albicans* in pit and fissure caries. Therefore, investigations on the microbiology of occlusal caries should always include yeasts.

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Disclosure Statement

There are no conflicts of interest for any of the authors involved in this study.

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